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(71) Applicant: E.I. DU PONT DE NEMOURS AND COMPANY [US/US]; 1007 Market Street, Wilmington, DE 19898 (US).

(72) Inventors: HERBLIN, William, Fitts ; 3307 Altamont Drive, Wilmington, DE 19810 (US). NEVILLE, Mary, Elisabeth ; 143 Garvin Boulevard, Sharon Hill, PA 19079 (US).

(74) Agents: HORN, Margaret, A. et al.; E.I. du Pont de Nemours and Company, Legal/Patent Records Center, 1007 Market Street, Wilmington, DE 19898 (US).

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(54) Title: INHIBITION OF ANGIOGENESIS BY IL-1

(57) Abstract

There is disclosed the use of IL-1 and preferably IL-1β alone or in combination with additional anti-angiogenic factor(s) to inhibit angiogenesis thus providing therapeutic benefits in angiogenic mediated disease states, and particularly basic fibroblast growth factor mediated angiogenic disease states such as solid tumor, arthritis, atherosclerosis, psoriasis and diabetic retinopathy.

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TITLE

Inhibition of Angiogenesis by IL-1

FIELD OF THE INVENTION

This invention relates to the use of interleukin-1 (IL-1) and particularly IL-1β, as a means to inhibit angiogenesis and also the use of IL-1β to treat angiogenesis-mediated diseases in a mammal.

BACKGROUND OF THE INVENTION

IL-1α and β are proteins which are primarily produced by monocytes and macrophages (Dinarello (1987) Bull Inst. Pasteur 85:267; Oppenheim et al. (1986) Immunology Today 7:45). Other cells such as fibroblasts and endothelial cells can also produce IL-1 under certain conditions (Miossec et al. (1986) J. Immunol. 136:2486; Iribe et al. (1983) J. Exp. Med. 157:2190). IL-1 has been implicated in many biological functions including fever induction, T-cell activation, granulopoiesis, increased production of hormones, acute phase proteins and immunoglobulins and decreased production of iron-binding proteins and albumin. IL-1 also has been found to influence the breakdown and repair of tissue and bone by the stimulation of proteinases, collagenases and fibrinolysins.

IL-1α and β bind to the same receptors expressed on many different cells (Dower et al. (1985) J. Exp. Med. 162:501; Matsushima et al. (1986) J. Immunol. 136:4496) and affect the same pleomorphic biological functions (Dinarello (1987) Bull Inst. Pasteur 85:267). For example, the vascular and endothelial cell systems are greatly influenced by IL-1 (Dinarello (1987) Bull Inst. Pasteur 85:267; Oppenheim et al. (1986) Immunology Today 7:45). Factors from macrophages, such as tumor necrosis factor (TNF) (Schroder et al. (1987) Proc. Natl. Acad. Sci. USA 84:5277) and prostaglandins (Ziche et al. (1982) J. of Natl. Cancer Inst. 69:475), have been reported to stimulate the growth of endothelial cells in vivo in a process called angiogenesis. More recently recombinant human IL-1α and β have been reported to

be angiogenic in two different in vivo models (Mahadevan et al. (1989) Cancer Res. 49:415; Predergast et al. (1987) Fed. Proc. 46:1200).

Angiogenesis is the process of new capillary formation which usually involves the directional mobilization of endothelial cells, cellular proliferation, canalization and production of periendothelial stroma (Ausprunk and Folkman (1977) Microvasc. Res. 14:53). Angiogenesis may be required in pathological conditions such as the growth of solid tumors (Folkman (1986) Cancer Res. 46:467), rheumatoid arthritis (Matsubara and Ziff (1987) J. Clin. Invest. 79:1440), atherosclerosis (Klagsbrun and Edelman (1989) Arteriosclerosis 9:269) and ocular neovascularization (Folkman (1986) Cancer Res. 46:467). In addition, angiogenesis has been described as the dominant pathology in the following diseases: retrolental fibroplasia, hemangiomas, angiofibromas, psoriasis (Folkman (1987) Science 235:442-47); and pulmonary capillary hemangiomatosis (Folkman (1989) NEJM 320:1211-12). Thus, the inhibition of angiogenesis may be therapeutically beneficial in these and related angiogenesis-mediated disease states (Folkman (1986) Cancer Res. 46:467; Matsubara et al. (1987) J. Clin. Invest 79:1440; Klagsbrun et al. (1989) Arteriosclerosis 9:269).

Angiogenesis requires factors, including proteins, produced by cells to orchestrate the process. Several of these proteins (Schroder et al. (1987) Proc. Natl. Acad. Sci. USA 84:5277; Thomas et al. (1985) Proc. Natl. Acad. Sci. USA 82:6409; Jaye et al. (1986) Science 233:541) have been identified and purified and the genes cloned and expressed using recombinant DNA technology (Thomas et al. (1985) Proc. Natl. Acad. Sci. USA 82:6409; Jaye et al. (1986) Science 233:541). In particular, the fibroblast growth factor (FGF) is a potent angiogenic factor (Thomas et al. (1985) Proc. Natl. Acad. Sci. USA 82:6409; Abraham et al. (1986) Science 233:545). There are two forms of FGF, basic (bFGF) (Abraham et al. (1986) Science 233:545) and acidic (aFGF) (Thomas et al. (1985) Proc. Natl. Acad. Sci. USA 82:6409). Both forms

bind to the same receptors (Olwin et al. (1986) Biochemistry 25:3488; Neufeld et al. (1987) J. Biol. Chem. 261:5631). Both forms stimulate angiogenesis (Thomas et al. (1985) Proc. Natl. Acad. Sci. USA 82:6409; Abraham et al. (1986) Science 233:545) and share approximately 30% amino acid homology with IL-1 (Thomas et al. (1985) Proc. Natl. Acad. Sci. USA 82:6409; Abraham et al. (1986) Science 233:545). However, it has been found that IL-1 does not compete for FGF binding (Chin and Herblin, unpublished data). Although IL-1α and β have both been reported to be angiogenic in two different in vivo assays (Mahadevan et al. (1989) Cancer Res. 49:415; Predergast et al. (1987) Fed. Proc. 46:1200) it has been found that in certain assays wherein FGF is a factor in angiogenesis, IL-1 may inhibit angiogenesis. Thus, in those pathological conditions which require angiogenesis, and particularly those in which FGF is a factor in stimulating angiogenesis, such as growth of solid tumors, rheumatoid arthritis, atherosclerosis, psoriasis and ocular neovascularization (such as diabetic retinopathy or neovascular glaucoma) IL-1 may inhibit angiogenesis and thereby provide a therapeutic benefit in the treatment of these conditions.

SUMMARY OF THE INVENTION

This invention relates to the use of IL-1 and preferably IL-1β in the treatment of angiogenesis-mediated and/or bFGF-mediated disease states in a mammal, and more particularly relates to administering to the mammal an angiogenesis-inhibiting amount of IL-1β alone or in combination with other anti-angiogenic factors.

We have discovered that IL-1β inhibits angiogenesis in vivo in an intradermal murine tumor model. The ability of IL-1 to inhibit angiogenesis in vivo may have therapeutic applications in diseases or conditions where abnormal angiogenesis is part of the pathology. This discovery provides novel therapies to treat such pathological conditions as growth of solid tumors, rheumatoid arthritis, atherosclerosis, fibroplasia, hemangioma, neovascular glaucoma, psoriasis, and diabetic retinopathy, which require

angiogenesis for their progression. IL-1 may also have application in the prevention of atheromatous plaque in cardiovascular disease.

DETAILED DESCRIPTION OF THE INVENTION

IL-1 inhibited the proliferation of human endothelial cells in vitro in a dose-dependent manner. In vivo, IL-1, when given systemically, inhibited the growth of B16 melanoma in syngeneic mice. This inhibition in tumor growth was related to a decrease in number of endothelial cells within the tumor and the loss of sites for radiolabeled bFGF to bind to the endothelial cells within the tumor. Basic FGF is a potent stimulator of angiogenesis (Klagsbrun and Shing (1985) Proc. Natl. Acad. Sci. USA 82:805; Gospodarowicz et al. (1986) Endocrinology 118:82) and binding of bFGF to its receptors stimulates growth of endothelial cells (Schreiber et al. (1985) Proc. Natl. Acac. Sci. USA 82:6138; Friesel et al. (1986) J. Biol. Chem. 261:7581; Huang and Huang (1986) J. Biol. Chem. 261:9568). Therefore the mechanism of action of IL-1 on angiogenesis may include its capacity to decrease the number of receptors for bFGF on the endothelial cells, thus limiting bFGF stimulation of growth.

In contrast to previous reports (Mahadevan et al. (1989) Cancer Res. 49:415; Predergast et al. (1987) Fed. Proc. 46:1200) that IL-1 induces angiogenesis, we have found that IL-1β causes an inhibition of angiogenesis, using the B16 intradermal murine tumor model (Kreisle and Erchler (1988) J. Natl. Cancer Inst. 80:849). It has been reported earlier that a series of amino-terminal muteins of human IL-1 have B16 tumor inhibitory activity (Huang et al. (1987) FEBS Letters 223:294-298). However, this reference did not discuss or suggest any mechanism for inhibition of B16 tumors. We have found that IL-1α and β may inhibit angiogenesis through the down-regulation of the cellular receptor of bFGF. For example, it was discovered that the addition of IL-1α or β to cultures of rabbit chondrocytes, which were stimulated by bFGF, inhibited the growth of the chondrocytes (Example 1).

IL-1 decreased the number of bFGF receptors of the chondrocytes while decreasing the Kd (increasing the affinity) of the remaining receptors. We also tested the effects of IL-1β in an intradermal murine B16 melanoma model (Example 2). IL-1β inhibited tumor growth in a dose-dependent manner when given locally (intratumorally) or systemically (intraperitoneally, intramuscularly or intradermally). It was observed that after 1 or 2 injections of IL-1β the tumor mass contained less blood.

Example 3 shows that a monoclonal antibody which neutralized the activity of bFGF inhibits the growth of B16 tumors. Example 4 shows that the blood volume of IL-1β-treated tumors was significantly less than sham-treated tumors and that the number of endothelial cells from IL-1β-treated tumors was less than the number of endothelial cells from sham-treated tumors. Finally, bFGF-stimulated growth of human endothelial cells was inhibited by human IL-1β (Example 5). These results suggest that IL-1β inhibits the growth of endothelial cells and angiogenesis in the B16 melanoma model, and thereby inhibits the B16 melanoma, which, in part, requires bFGF and bFGF-mediated angiogenesis for its growth.

The mechanism of inhibition of angiogenesis in the B16 tumors may be similar to the situation in the rabbit chondrocyte model, i.e., a decrease in the number of bFGF cell surface receptors. Our findings indicate that there is a decrease in the binding of ^{125}I-labelled bFGF to B16 tumor sections from mice treated in vivo with IL-1β. The binding of ^{125}I-labelled bFGF is associated primarily with the endothelial cells within B16 tumors and not the B16 melanoma cells. Therefore IL-1β may regulate the receptors of bFGF on endothelial cells in vivo and this down-regulation in bFGF receptor levels may inhibit the angiogenic response to bFGF and may contribute to the IL-1-mediated inhibition of angiogenesis and tumor growth.

Although B16 mouse melanoma cells in culture do not express high affinity binding sites for FGF, sections of B16 tumors grown in syngeneic mice show numerous patches of high affinity sites.

Using immunostaining, we have demonstrated that these patches lie over areas that stain for Factor VIII, a marker for endothelial cells. This suggests that the FGF sites reside on the endothelial cells of the invading capillaries.

Example 1

IL-1β Decreases the Number of bFGF Receptors and Blocks the Mitogenic Effect of bFGF on Rabbit Chondrocytes

The effect of IL-1 and bFGF on rabbit articular chondrocytes, which are target cells for both proteins, was examined. FGF has been shown to promote chondrocytes growth (Prins (1982) Arthritis Rheum. 25:1217-1227) and proteoglycan synthesis (Cuevas et al. (1988) Biochem. Biophys. Res. Commun. 156:611-618; Kato and Gospodarowicz (1984) J. Cell. Physiol. 120:354-363) in vitro. We discovered that IL-1β and bFGF had opposite effects on chondrocyte cell growth and that IL-1β, in fact, completely blocked the mitogenic effect of bFGF. Pretreatment of chondrocytes with IL-1β was found to induce a down-regulation of the number of functional bFGF high affinity receptors.

Materials. 12-week old Male New Zealand White rabbits were obtained from Hare-Marland (Hewitt, NJ). Tissue culture plastics were from Costar (Cambridge, MA). Dulbeccos's modified Eagle's medium (DMEM) was from Hazleton (Denver, PA) and fetal bovine serum (FBS) was obtained from Hyclone (Logan, UT). Human serum albumin (HSA) was purchased from Armour Pharmaceuticals (Kankakee, IL). Penicillin, streptomycin, and trypsin-EDTA were from Gibco (Grand Island, NY). Trypsin, hyaluronidase and actinomycin D were from Sigma (St. Louis, MO). Collagenase was from Worthington (Freehold, NJ). Recombinant human IL-1β was produced in our laboratory (Huang et al. (1987) Mol. Biol. Med. 4:169-181) with a specific activity of 1×10^7 units/mg protein. Recombinant human bFGF was provided by Synergen (Boulder, CO).

Cell culture. Articular cartilage was dissected from the knee joints of 12-week old male New Zealand White rabbits. A single cell suspension of chondrocytes was obtained by sequential

enzymatic digestion using a modified protocol of Chin and Lin (Chin and Lin (1988) Arthritis Rheum. 31:1290-1296).

Effect of IL-1β on chondrocyte growth. Cultures used for these experiments were in logarithmic growth phase. Cultures were treated with IL-1β, bFGF, or both, in DMEM and 5% FBS after the cells had been allowed to settle and attach for 48-72 hours in 6-well culture plates. At the appropriate times after addition of IL-1β, bFGF, or both, the cells were detached with a trypsin-EDTA (0.05% trypsin, 0.53 mM EDTA) solution. Cells were washed twice with phosphate buffered saline (PBS), pH 7.4. Cell counts were made using a coulter counter.

Table 1 shows that bFGF at 10 ng/mL is a mitogen for rabbit articular chondrocytes. IL-1β at 10 units/mL had the expected negative effect on growth (Huang et al. (1987) Mol. Biol. Med. 4:169-181). Surprisingly, when IL-1β and bFGF were co-administered, the mitogenic effect of bFGF was entirely blocked. At both time points the inhibitory effect of IL-1β appeared to have effectively negated all the mitogenic activity of bFGF.

Table 1

IL-1β Inhibits Growth of bFGF-stimulated Rabbit Chondrocytes

	Cell #/Well (x 10^5)	
	Day 3	Day 4
Control	5.08 (± 0.06)	6.37 (± 0.08)
IL-1 (10 unit/mL)	3.87 (± 0.03)	5.35 (± 0.06)
bFGF (10 ng/mL)	7.07 (± 0.25)	9.79 (± 0.4)
bFGF (10 ng/mL) + IL-1 (10 units/mL)	3.78 (± 0.08)	6.11 (± 0.14)

The bFGF receptor binding assay. bFGF was iodinated using a modification of the lactoperoxidase procedure reported by Sonnenfeld et al. (Sonnenfeld et al. (1985) J. Neuroscience 5:1717-1728). The binding assay for bFGF was performed with [^{125}I]bFGF on confluent chondrocyte cultures in 24-well culture

plates using the procedure reported by Moscatelli for baby hamster kidney cells (Moscatelli (1987) J. Cell. Physiol. 131: 123-130). Briefly, the cells were incubated with 20-40 pM [^{125}I]bFGF in DMEM containing 0.15% gelatin and 25 mM HEPES at pH 7.5 for two hours at 0-4°C. At the end of the incubation, the medium was removed and the cells washed with cold PBS to determine free ligand. The cells were washed twice with 2 M NaCl in 20 mM HEPES at pH 7.5 to remove low affinity binding and then with 2 M NaCl in 20 mM sodium acetate at pH 4.0 to collect high affinity binding. The average number of cells/well on the 24-well plates was 2.5 x 10^5.

The binding of bFGF to both high and low affinity sites on chondrocytes was demonstrated by the addition of increasing concentrations of unlabeled bFGF to the wells. The counts removed by 2 M NaCl at pH 7.5 represented low affinity binding, which was displaced by the unlabeled bFGF with an IC50 of 890 nM. The remaining counts, removed by 2 M NaCl at pH 4.0, represented high affinity binding that was displaced with an IC50 of 120 pM.

The low affinity binding was variable and estimates of the binding parameters by non-linear regression ranged from 100-600 nM for Kd and from 21-250 X 10^6 sites/cell. The high affinity binding was much more reproducible and yielded estimates of 59 ± 4.2 pM for Kd and 8.72 ± 0.24 fmoles/well (21,000 sites/cell).

Modulation of bFGF receptors by IL-1β. Confluent chondrocyte cultures in 24-well plates were treated with 100 units/mL IL-1β in DMEM and 10% FBS for 17 hours at 37°C. The cells were then washed with DMEM and the receptor binding assay for bFGF was performed as outlined.

When confluent chondrocytes were treated with 100 units/mL of IL-1β for 17 hours at 37°C, a pronounced change in bFGF binding was observed. IL-1β caused an increase in the affinity of the high affinity receptor and a concomitant reduction in the number of functional binding sites/cell (Table 2).

Table 2

IL-1β Decreases the Number and Increases the Affinity of bFGF Receptors on Rabbit Chondrocytes

IL-1β Treatment	Kd (pM)	bFGF Bound (fmol/well)	bFGF-binding sites/cell
-	59.0 ± 4.2	8.72 ± 0.24	21,000
+	21.5 ± 3.1	2.96 ± 0.11	7,200

High affinity binding of [^{125}I]bFGF removed by the pH 4.0 washes was determined on chondrocytes that had been treated overnight (17 hours) with 100 units/mL IL-1β. Results shown represent mean of duplicate wells from two separate experiments (each experiment included 48 wells).

IL-1β, in concentrations up to 17,000 units/mL did not affect the binding of bFGF when added directly to the binding assay. Similar, but less pronounced changes were also observed for the low affinity bFGF binding sites.

We have shown that the number of high affinity receptors for bFGF on rabbit chondrocytes was down-regulated by IL-1β from 21,000 to 7,200 receptor sites/cell. The present results suggest that IL-1 can inhibit the biological action of bFGF, in part, by down-regulation of the level of functional bFGF high affinity receptors on a cell.

Example 2

Inhibition of Growth of B16 Melanoma In Vivo by IL-1β

Animals. Female C57BL/6 mice (7-12 weeks old, viral antibody free) were purchased from Charles River.

Tumor. B16 melanoma cells were obtained from the National Cancer Institute (NCI) Tumor Repository, Frederick, MD, and were negative for viruses by mouse antibody production (MAP) testing and were mycoplasma free. The cells were maintained in vitro in

RPMI 1640 (Gibco, Grand Island, NY) supplemented with 10% FCS (Hyclone, Logan, UT).

IL-1 Preparations. The recombinant human and murine IL-1β were prepared through the use of an expression system in E. coli and purified by the means of ion exchange and gel filtration chromatography (Huang et al. (1988) Mol. Biol. Med. 4:169; Huang et al. (1988) J. Immunol. 140:3838). The biological activity, evaluated in the murine thymocyte assay (Lachman et al. (1985) Methods Enzymol. 116:467), was between 1-2 X 10^7 units/mg for human IL-1β and 7-10 X 10^7 units/mg for murine IL-1β. The endotoxin levels were 3-10 ng/mg of protein as determined by the QCL 1000 Limulus amebocyte assay (MA Bioproducts, Walkerville, MD). IL-1β was diluted with D-PBS (Gibco) containing 0.5% normal C57BL/6 serum).

Tumor Therapy Experiments. The tumor cells used for transplantation were resuspended in PBS at the concentration of 5 X 10^6 cells/mL. Mice were injected intradermally on the ventral surface with 0.1 mL of the cell suspension. At this concentration, the tumor take was 100%. Animals with tumors weighing approximately 200 mg were used in these experiments. 100 μL of IL-1β were administered intramuscularly (IM) in the left or right flank or intratumorally (ITU). Control mice were injected with the diluent according to the same schedule as the IL-1β-treated mice. Tumor weight (mg) was calculated by the formula 0.5 X (W^2 X L^2), where W and L represent the width (mm) and length (mm) of the tumor. Percent inhibition of tumor growth = (1-T/C) 100%, where T and C represent the mean tumor weight of the treated and control groups. The Student's t-test was used for statistical analysis using the Balance software program (Elsevier Scientific Software, Amsterdam, Netherlands).

We found that IL-1β inhibits the growth of B16 melanoma in vivo in a dose-dependent manner when given intratumorally (Table 3 and 4). IL-1β also significantly inhibited the growth of B16 melanoma when given either intradermally or intramuscularly (Table 5). These results indicate that IL-1β has antitumor properties.

Table 3

Inhibition of Growth of B16 Melanoma by Murine IL-1β is Dose-dependent

	Daily Dose (μg/mouse)[a]	Tumor Weight (mg)[b]	Inhibition (%)[c]
Experiment A	0	1246 ± 346	--
	0.1	1022 ± 293	18
	0.3	845 ± 346	32
	1	660 ± 198 **	47
	3	286 ± 47 ***	77
	5	252 ± 58 ***	80
Experiment B	0	1785 ± 648	--
	3	362 ± 222 **	80
	5	176 ± 89 **	90
	7	133 ± 162 **	93

a C57BL/6 mice (N=7) were transplanted ID with B16 melanoma cells on day 0. IL-1β was administered ITU once a day for 7 days from day 9 to day 15.

b Mean ± S.D. Statistically significant differences from the control groups: ** $p < 0.01$ and *** $p < 0.001$.

c (1 - mean tumor of test group/mean tumor weight of control group) x 100 (%). Inhibition was calculated on day 16.

Table 4

Dose-dependent Antitumor Effect of Human IL-1β on B16 Melanoma[a]

	Daily Dose (μg/mouse)	Tumor Weight (mg)[b]	Inhibition (%)[c]
Experiment A	0	1345 ± 314	--
	1	863 ± 92 **	36
	3	596 ± 76 ***	56
	10	147 ± 87 ***	89
	20[d]	89 ± 84 ***	93
Experiment B	0	1785 ± 648	--
	15	70 ± 70 ***	96

a C57BL/6 mice (N=7) were transplanted ID with B16 melanoma cells on day 0. IL-1β was administered ITU once a day for 7 days from day 8 to day 14. Inhibition was calculated on day 15.

b Mean ± S.D. Statistically significant differences from the control groups: ** $p < 0.01$ and *** $p < 0.001$.

c (1 - mean tumor weight of test group/mean tumor weight of control group) x 100 (%).

d Injected for 4 days from day 8 to day 11. This dose was toxic and resulted in 2 mortalities.

Table 5

Comparison of the Antitumor Effect of Human IL-1β on B16 Melanoma by Various Routes of Injection

	Route Of IL-1β Injection[a]	Tumor Weight (mg)[b]	Inhibition (%)[c]
Experiment A	0	1181 ± 209	--
	ID	687 ± 454 *	42
	IM	610 ± 153 ***	48
Experiment B	0	1324 ± 500	--
	IP	960 ± 300	27
	ID	667 ± 247 *	50

[a] C57BL/6 mice (N=6) were transplanted ID with B16 melanoma cells on day 0. IL-1β was administered by various routes once a day for 7 days from day 10 to day 14 at a daily dose of 30 μg/mouse. Inhibition was calculated on day 15.

[b] Mean ± S.D. Statistically significant differences from the control groups: * $p < 0.05$ and *** $p < 0.001$.

[c] (1 - mean tumor weight of test group/mean tumor weight of control group) x 100 (%).

Example 3

B16 Melanoma Growth Is Inhibited by bFGF-neutralizing Monoclonal Antibodies

The effect of bFGF-specific mAbs was also tested in the mouse B16 melanoma model. Female C57BL/6 mice (N=5) weighing 18 to 20 g were injected intradermally (ID) with 1×10^5 B16 melanoma cells (ATCC, Rockville, MD) on day 0. On day 10, when the tumors were approximately 300 mg, DG2 monoclonal antibody to bFGF (5 μg/injection), MOPC-21, a control murine monoclonal antibody (5 μg/injection), murine recombinant IL-1β (3 μg/injection) or vehicle (PBS) were injected intratumorally (ITU) for 5 consecutive days. Tumors were measured on day 13 and 17. The

results are shown in Table 6. As shown in Table 6, DG2 and IL-1β inhibited the growth of the B16 melanoma tumor in the mouse. DG2 monoclonal antibody is described in the commonly assigned application of Reilly et al. DG2 is produced by hybridoma cell designated DG2 which is on deposit in ATCC under accession number HB10202. The results show that DG2, a monoclonal antibody which inhibits the in vivo angiogenic response in a rat kidney capsule angiogenesis model, as described in Reilly et al., also inhibits the growth of B16 melanoma in vivo. The results suggest that growth of B16 melanoma is dependent, in part, on bFGF-mediated angiogenesis.

Table 6

B16 Melanoma Growth is Dependent on bFGF:
A Monoclonal Antibody That Neutralizes
bFGF Inhibits B16 Growth

Treatment (ITU)	Mean Tumor Weight (mg + S.D.) (N=5) Days Following Treatment 0	3	7
PBS	293 ± 98	446 ± 356	1052 ± 268
IL-1β	302 ± 96 (0)	245 ± 242 (45)	263 ± 92 (75)
MOPC-21	304 ± 93 (0)	501 ± 321 (0)	1011 ± 104 (0)
DG2	302 ± 89 (0)	300 ± 259 (40)	568 ± 211 (44)

The percent inhibition with respect to the untreated tumor is given in parentheses.

Example 4

Inhibition of Angiogenesis by IL-1β

IL-1β decreases blood volume and the number of endothelial cells within B16 melanomas. Angiogenesis is the process of new capillary formation which involves the directional mobilization, cellular proliferation, and canalization of endothelial cells. Angiogenesis requires factors including proteins produced by cells to orchestrate these processes. One of these proteins is

bFGF. An antibody that neutralizes bFGF's angiogenic activities also inhibits the growth of B16 melanoma (Table 6), which indicates that B16 melanoma requires, in part, bFGF-mediated angiogenesis for its growth. If IL-1β is inhibiting the growth of B16 tumor by inhibiting angiogenesis then there should be less blood volume and fewer endothelial cells within the tumors of IL-1β treated mice.

The following experiment was done to determine blood volume of tumors after treatment with IL-1β. C57BL/6 mice were injected intradermally with B16 cells (5×10^5). Ten days later, when the tumors were approximately 150 mg, murine IL-1β (10 μg/mouse/day) or PBS was injected intradermally for 3 consecutive days. On the fourth day the mice were bled from the retro-orbital plexus and sacrificed. Tumors were excised, weighed and homogenized in PBS at 50 mg/mL. The amount of hemoglobulin (Hg) per mL of blood was determined for each mouse using a Sigma™ hemoglobulin assay. One mL of tumor homogenate was washed 3 X in PBS by centrifugation. After the final wash the cells of the tumor homogenate were lyzed with one mL of Triton X 100. Cellular debris was removed by centrifugation and hemoglobulin concentration of the lysates was determined using the Sigma™ hemoglobulin assay. The blood volume/gram of tumor was calculated using this formula.

Blood volume (mL)/gram of tumor = mg of Hg/gr of tumor divided by mg of Hg/mL of blood. The data in Table 7 shows that IL-1β does significantly decrease the volume of blood within the tumor compared to sham-treated control mice after only 3 daily systemic injections. These results suggest that IL-1β may inhibit the growth of the neovasculature within the tumor resulting in less blood volume.

Table 7

IL-1β Decreases the Volume of Blood in B16 Melanoma

Treatment (intramuscular)	Weight of Tumor (mg)	Blood Volume (mL/g of tumor)
PBS	180	0.35
	240	0.36
	200	0.42
	222	0.49
mean ± S.D.	210 ± 26	0.40 ± 0.06
IL-1β	360	0.28
	100	0.33
	166	0.27
	80	0.30
mean ± S.D.	176 ± 127	0.29 ± 0.2, p=.02

The following experiment was done to determine whether IL-1 decreases the number of endothelial cells within B16 tumors. C57BL/6 mice were injected intradermally with B16 cells (5×10^5) and 10 days later, when the tumors were approximately 350 mg, treatment was started. A group of 5 mice received intradermal daily injections of either PBS or IL-1β (10 μg/injection) for 3 consecutive days. On day 4 the mice were injected intratumorally with acetylated low density lipoprotein (LDL) labeled with 1,1'dioctadecyl-1-3,3,3',3'-tetramethyl-iodo-carbocyanine perchlorate (Biomedica Technologies Inc., Stoughton, MA), which specifically labels endothelial cells (Voyta et al. (1985) J. Cell. Biol. 99:81A; Eshu et al. (1985) Biochem. Biophys. Res. Commun. 133:554). Two hours after the injection of acetylated LDL, the mice were sacrificed and the tumors were removed, weighed and homogenized at 50 mg of tumor/mL of PBS. Two 1 mL samples of each homogenate were washed with 10 mL of PBS 3-times. After the final wash, one sample was resuspended in 1 mL of PBS and a total cell count was determined using a hemocytometer and microscope without regard to viability. Cells were resuspended at 5×10^5/mL in PBS containing 0.1% sodium azide and 5% FCS.

The percentage of positively stained cells were determined using flow cytometry (FACS analyzer, Becton and Dickinson, Mountain View, CA). The other samples were lysed with a detergent, Triton X100, and cellular debris was removed by centrifugation. The concentration of protein/mL of homogenate was determined for each homogenate using BCA protein assay (Pierce, Rockford, IL). The number of endothelial cells/mg of total cellular protein was determined from these assays using the formula: # of endothelial cells/mg of protein = # of stained cells X number of total cells/mg of total protein. The results of this experiment are shown in Table 8. As shown in Table 8, systemic treatment of mice with IL-1β decreased the number of endothelial cells within the B16 tumor by 43%. These results suggest that IL-1β inhibits angiogenesis occurring within B16 melanomas.

In another experiment (Table 9) endothelial cells were visually identified using a fluorescence microscope (Ziess) instead of FACS analysis. The protocol of the preceding experiment was used with the exception that IL-1β was given for 2 consecutive days. A reduction (41%) in the number of endothelial cells was observed when IL-1β was given intradermally to tumor-bearing mice compared to the sham-treated controls. These results, presented in Tables 8 and 9, suggest that IL-1β inhibits angiogenesis by inhibiting the proliferation of endothelial cells within the tumor.

Table 8

IL-1β Decreases the Number of Endothelial Cells within B16 Melanomas

Treatment	Cells X 10^6/mg of Protein	% Endothelial Cells	Endothelial Cells 10^6/mg of Protein
PBS	2.6	49	1.3
	1.3	45	.6
	3.8	61	2.3
	2.2	50	1.1
	2.1	47	1.9
mean ± S.D.	2.8 ± 1.2	50 ± 6	1.4 ± .7
IL-1β	1.5	50	0.75
	2.2	38	0.84
	3.1	36	1.11
	2.2	35	0.77
	2.1	22	0.46
mean ± S.D.	2.2 ± 5.7*	36 ± 10 **	0.78 ± .2**

* Not significantly different from PBS control at the 95% confidence limit.

** Significantly different from the PBS control at the 95% confidence limit.

Table 9

IL-1β Decreases the Number of Endothelial Cells Within B16 Melanoma

Treatment	Weight of Tumors (g ± S.D.)	Number of Endothelial Cells/g of Tumor (mean ± S.D.)	% Inhibition of Endothelial Cells
PBS (N=3)	.24 ± .08	4.3 ± 0.17 X 10^7	--
IL-Iβ (N=2)	.28 ± .08	2.5 ± 0.07 X 10^7	41

Example 5

IL-1β Inhibits bFGF-mediated Growth of Human Endothelial Cells in vitro

Human endothelial cells from saphenous veins (1 X 10^4) were cultured on day 1 in RPMI 1640 (Gibco, Grand Island, NY)

supplemented with 0.5% fetal calf serum and 100 ng/mL of bFGF, with and without IL-1β, in 48-well multi-well plates (Costar, Cambridge, MA), and their growth was monitored by cell counts on days 3, 5, and 7 (Table 10).

Table 10

IL-1β Inhibits bFGF-mediated Growth of Human Endothelial Cells in vitro

Treatment	Day	Number of Endothelial Cells/Well	% Inhibition of Growth
None	3	1.1×10^4	--
IL-1β (100 ng/mL)	3	1.1×10^4	0
IL-1β (1000 ng/mL)	3	0.9×10^4	9
None	5	1.5×10^4	--
IL-1β (100 ng/mL)	5	0.9×10^4	40
IL-1β (1000 ng/mL)	5	0.8×10^4	47
None	7	3.8×10^4	--
IL-1β (100 ng/mL)	7	1.5×10^4	61
IL-1β (1000 ng/mL)	7	1.5×10^4	61

Endothelial cells were treated with 100 ng/mL bFGF on day 1. Human endothelial cells were obtained from Dr. T. Watt, E. I. du Pont de Nemours and Company, Du Pont Experimental Station, Wilmington, DE.

As shown in Table 10, IL-1β inhibited the growth of the human endothelial cells. IL-1β was not directly toxic to endothelial cells since the number of viable cells in the presence of IL-1β was equal to or greater than the number of cells plated on day 1.

These results suggest that IL-1β may indeed inhibit angiogenesis in vivo by blocking the proliferation of endothelial cells.

Thus it has been shown that B16 growth is under the control of angiogenic factors, including bFGF, that IL-1β decreases the blood volume within B16 tumors and decreases the number of endothelial cells within the tumor and that IL-1β inhibits the proliferation of human endothelial cells in vitro.

Dosage and Administration

IL-1 can be administered as a sterile, nonpyrogenic parenteral solution. The aqueous parenteral vehicle could be, for example, Sterile Water for Injection USP, 0.9% Sodium Chloride for Injection USP or 5% Dextrose Injection USP. Pharmaceutical compositions of the present invention comprise an effective amount of purified IL-1β and a pharmaceutically acceptable carrier such as listed above, and optionally additional excipients, such as preservatives and buffers as known to those skilled in the art of pharmaceutical formulations. See, for example, Remington's Pharmaceutical Sciences, 17th Edition, Osol (1985), a standard reference in the field. IL-1 may be prepared in a stable formulation ready for administration, or for dilution in an appropriate intravenous solution. Preferably, to increase product shelf life, IL-1 may be formulated, for example, as a sterile lyophilized powder to be reconstituted aseptically as a buffered solution. In addition, IL-1 may be formulated in combination with at least one additional antiangiogenic factor, such as the DG2 monoclonal antibody along with a pharmaceutically acceptable carrier.

As is known to those skilled in the art, individual patient dosages will vary depending upon known factors such as the pharmacodynamic characteristics of the particular active ingredient, and its mode and the route of administration; the age, health, and weight of the recipient; the nature and extent of symptoms; the kind of concurrent treatment and frequency of treatment; and the effect desired.

WHAT IS CLAIMED IS:

1. A method of treating an angiogenesis-mediated disease state in a mammal comprising administering to the mammal an angiogenesis-inhibiting amount of IL-1.

2. A method of Claim 1 wherein the IL-1 is IL-1β.

3. A method of Claim 1 wherein the angiogenesis-mediated disease is mediated in whole or in part by fibroblast growth factor.

4. A method of Claim 2 wherein the angiogenesis-mediated disease is mediated in whole or in part by fibroblast growth factor.

5. A method of Claim 3 wherein the fibroblast growth factor is basic fibroblast growth factor.

6. A method of Claim 4 wherein the fibroblast growth factor is basic fibroblast growth factor.

7. A method of Claim 1 wherein the disease state is a solid tumor.

8. A method of Claim 2 wherein the disease state is a solid tumor.

9. A method of Claim 3 wherein the disease state is a solid tumor.

10. A method of Claim 4 wherein the disease state is a solid tumor.

11. A method of Claim 5 wherein the disease state is a solid tumor.

12. A method of Claim 6 wherein the disease state is a solid tumor.

13. A method of Claim 1 wherein the disease state is arthritis.

14. A method of Claim 2 wherein the disease state is arthritis.

15. A method of Claim 3 wherein the disease state is arthritis.

16. A method of Claim 4 wherein the disease state is arthritis.

17. A method of Claim 5 wherein the disease state is arthritis.

18. A method of Claim 6 wherein the disease state is arthritis.

19. A method of Claim 1 wherein the disease state is atherosclerosis.

20. A method of Claim 2 wherein the disease state is atherosclerosis.

21. A method of Claim 3 wherein the disease state is atherosclerosis.

22. A method of Claim 4 wherein the disease state is atherosclerosis.

23. A method of Claim 5 wherein the disease state is atherosclerosis.

24. A method of Claim 6 wherein the disease state is atherosclerosis.

25. A method of Claim 1 wherein the disease state is psoriasis.

26. A method of Claim 2 wherein the disease state is psoriasis.

27. A method of Claim 3 wherein the disease state is psoriasis.

28. A method of Claim 4 wherein the disease state is psoriasis.

29. A method of Claim 5 wherein the disease state is psoriasis.

30. A method of Claim 6 wherein the disease state is psoriasis.

31. A method of Claim 1 wherein the disease state is diabetic retinopathy.

32. A method of Claim 2 wherein the disease state is diabetic retinopathy.

33. A method of Claim 3 wherein the disease state is diabetic retinopathy.

34. A method of Claim 4 wherein the disease state is diabetic retinopathy.

35. A method of Claim 5 wherein the disease state is diabetic retinopathy.

36. A method of Claim 6 wherein the disease state is diabetic retinopathy.

37. A method of treating an angiogenesis-mediated disease state in a mammal comprising administering to the mammal an angiogenesis-inhibiting amount of IL-1β in combination with at least one additional anti-angiogenic factor or derivative thereof.

38. A method of Claim 37 wherein the angiogenesis-mediated disease is mediated in whole or in part by fibroblast growth factor.

39. A method of Claim 38 wherein the fibroblast growth factor is basic fibroblast growth factor.

40. A method of Claim 37 wherein the additional anti-angiogenic factor is a monoclonal antibody, or an antigen-binding fragment thereof, which inhibits basic fibroblast growth factor-mediated angiogenesis in a mammal.

41. A method of Claim 38 wherein the additional anti-angiogenic factor is a monoclonal antibody, or an antigen-binding fragment thereof, which inhibits basic fibroblast growth factor-mediated angiogenesis in a mammal.

42. A method of Claim 39 wherein the additional anti-angiogenic factor is a monoclonal antibody, or an antigen-binding fragment thereof, which inhibits basic fibroblast growth factor-mediated angiogenesis in a mammal.

43. A pharmaceutical composition comprising a suitable pharmaceutical carrier and a combination of a therapeutically effective amount of IL-1 and at least one additional anti-angiogenic factor.

44. A pharmaceutical composition comprising a suitable pharmaceutical carrier and a therapeutically effective amount of IL-1 and a monoclonal antibody to human bFGF, or an antigen-binding fragment thereof, which inhibits bFGF-mediated angiogenesis in a mammal.

45. A pharmaceutical composition of Claim 43 or 44, wherein the IL-1 is IL-1β.

INTERNATIONAL SEARCH REPORT

International Application No. PCT/US90/07220

I. CLASSIFICATION OF SUBJECT MATTER (if several classification symbols apply, indicate all) [6]

According to International Patent Classification (IPC) or to both National Classification and IPC

IPC(5): A61K 45/05; A01N 37/18; A61K 37/00

U.S. Cl.: 424/85.1; 424/85.2; 514/2

II. FIELDS SEARCHED

Minimum Documentation Searched [7]

Classification System	Classification Symbols
U.S. Cl.	424/85.1; 424/85.2; 514/2

Documentation Searched other than Minimum Documentation to the Extent that such Documents are Included in the Fields Searched [8]

APS, Search terms: monoclonal antibody, anti-tumor therapy, fibroblast growth factor, antibody

III. DOCUMENTS CONSIDERED TO BE RELEVANT [9]

Category *	Citation of Document, [11] with indication, where appropriate, of the relevant passages [12]	Relevant to Claim No [13]
Y	US, A,4,444,744 (Goldenberg) 24 April 1984, See columns 22-24.	37-45
Y	US, A, 4,785,079 (Gospodarowicz et al.) 15 November 1988, See columns 1-2.	37-45
A	US, A, 4,816,436 (Jacobs) 28 March 1989, See entire document.	1-12,37-45
Y	US, A, 4,923,696 (Appel et al.) 08 May 1990, See column 9.	37-45
X Y	FEBS Letter, Volume 223, NO. 2, issued November 1987, Huang et al., "Muteins of Human Interleukin-1 that Show Enhanced Bioactivities", pages 294-298, see page 295 and Table 2.	1-12 37-45

* Special categories of cited documents: [10]

"A" document defining the general state of the art which is not considered to be of particular relevance

"E" earlier document but published on or after the international filing date

"L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)

"O" document referring to an oral disclosure, use, exhibition or other means

"P" document published prior to the international filing date but later than the priority date claimed

"T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention

"X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step

"Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art

"&" document member of the same patent family

IV. CERTIFICATION

Date of the Actual Completion of the International Search

20 February 1991

Date of Mailing of this International Search Report

19 MAR 1991

International Searching Authority

ISA/US

Signature of Authorized Officer

Richard Ekstrom

Form PCT/ISA/210 (second sheet) (July 1989)

III. DOCUMENTS CONSIDERED TO BE RELEVANT (CONTINUED FROM THE SECOND SHEET)

Category *	Citation of Document, with indication, where appropriate, of the relevant passages	Relevant to Claim No
Y	Science, Volume 221, No. 4612, issued 19 August 1983, Folkman et al., "Angiogenesis Inhibition and Tumor Regression Caused by Heparin or a Heparin Fragment in the Presence of Cortisone", pages 719-725, See Figures 3 and 4C.	37-39,43,45
Y	Japanese Journal of Cancer Research, Volume 77, No. 8, issued August 1986, Nakamura et al., "Antitumor Effect of Recombinant Human Interleukin 1 Alpha Against Murine Syngeneic Tumors", pages 767-773, See entire document.	1-12,37-45

FURTHER INFORMATION CONTINUED FROM THE SECOND SHEET

V. ☐ OBSERVATIONS WHERE CERTAIN CLAIMS WERE FOUND UNSEARCHABLE [1]

This international search report has not been established in respect of certain claims under Article 17(2) (a) for the following reasons:

1. ☐ Claim numbers , because they relate to subject matter [2] not required to be searched by this Authority, namely:

2. ☐ Claim numbers , because they relate to parts of the international application that do not comply with the prescribed requirements to such an extent that no meaningful international search can be carried out [3], specifically:

3. ☐ Claim numbers_____, because they are dependent claims not drafted in accordance with the second and third sentences of PCT Rule 6.4(a).

VI. ☒ OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING [2]

This International Searching Authority found multiple inventions in this international application as follows:

See Attachment

1. ☐ As all required additional search fees were timely paid by the applicant, this international search report covers all searchable claims of the international application.

2. ☐ As only some of the required additional search fees were timely paid by the applicant, this international search report covers only those claims of the international application for which fees were paid, specifically claims:

3. ☒ No required additional search fees were timely paid by the applicant. Consequently, this international search report is restricted to the invention first mentioned in the claims; it is covered by claim numbers:

1-12 and 37-45 (Telephone Practice)

4. ☐ As all searchable claims could be searched without effort justifying an additional fee, the International Searching Authority did not invite payment of any additional fee.

Remark on Protest

☐ The additional search fees were accompanied by applicant's protest.

☐ No protest accompanied the payment of additional search fees.

Serial No. PCT/US90/07220
Art Unit 189B

ATTACHMENT A (CONTINUATION OF PART VI)

VI. OBSERVATIONS WHERE UNITY OF INVENTION IS LACKING

This International Searching Authority found multiple inventions in this international application as follows:

I. A method of IL-1 administration to treat a solid tumor, classified in class 424, subclasses 85.1 and 85.2 (claims 7-12);

II. A method of IL-1 administration to treat arthritis, classified in class 424, subclasses 85.1 and 85.2 (claims 13-18);

III. A method of IL-1 administration to treat atherosclerosis, classified in class 424, subclasses 85.1 and 85.2 (claims 19-24);

IV. A method of IL-1 administration to treat psoriasis, classified in class 424, subclasses 85.1 and 85.2 (claims 25-30);

V. A method of IL-1 administration to treat diabetic retinopathy, classified in class 424, subclasses 85.1 and 85.2 (claims 31-36).

If no additional fee is received, generic claims 1-6, and 37-45 will be searched with the first group .

Serial No. PCT/US90/07220
Art Unit 189B

ATTACHMENT TO CHAPTER I PCT TELEPHONE MEMORANDUM
FOR LACK OF UNITY OF INVENTION

Detailed Reasons For Holding Lack of Unity of Invention

The claims of these five groups are directed to different inventions which are not so linked as to form a single general inventive concept. In particular, the claims of Groups I-V are directed to the use of IL-1 for the treatment of a number of diverse diseases; the search for any one group would not result in a complete and thorough search for any other group.